RADIOACTIVE PHOSPHORUS ACCUMULATION AND DISTRIBUTION IN TETRAHYMENA[1]

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The protozoan, *Tetrahymena pyriformis,* has in recent years become increasingly important in biochemical and physiological protozoology (Corliss, 1954). Mating types I and II of variety four were used in this investigation. First defined by Elliott and Hayes (1953) and then more extensively by Ray (1956), the cytological picture of these types appears to be representative of conjugation in all 45 mating types of the nine known varieties of *Tetrahymena* (Gruchy, 1954). According to Ray (1956) there are three prezygotic and three postzygotic divisions of the micronuclear derivatives during conjugation in this animal. Meiosis occurs during the first two prezygotic divisions and mitosis during the third prezygotic and all of the postzygotic divisions. Morphological reduplication is thought to occur in the crescent stage when chromosomal material elongates during prophase of the first meiotic division.

Since nothing was known concerning ion accumulation and distribution during cellular differentiation in conjugating protozoans, the intent of this investigation was to determine the influence of conjugation and its nuclear reorganization on phosphorus accumulation. The isotope P^{32} was chosen primarily because of the role of phosphorus in DNA synthesis. We were especially interested in delineating ion movements and accumulation just prior to the formation of the crescent stage, since it is here that an increased P^{32} uptake for DNA synthesis with chromosomal reduplication might be expected (Swift, 1950). Taylor (1952), working with *Tradescantia* and *Lilium* buds, found that periods of P^{32} incorporation into nuclei corresponded with the interphase period of chromosomal reduplication and DNA synthesis in both mitosis and meiosis. Other work with metazoan animal cells (Hull and Kirk, 1950) has also shown a correlation of P^{32} uptake with the rate of mitosis. A differential rate of P^{32} uptake may also result from other reasons than chromosomal incorporation. Since phosphorus plays an essential role in energy transfer processes, it can be assumed that the total energy required at certain stages of the tremendous nuclear reorganization during conjugation might involve ion uptake in significant quantity.

It was further decided to investigate P^{32} uptake in mass populations of the individual mating types in an attempt to evaluate better the comparative amounts of this substance used by each type while population interactions were present.

Finally, in an attempt to evaluate further physiological differences between the

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two mating types, the quantitative intracellular distribution of phosphorus was studied. Thus, the distribution of phosphorus in the acid-soluble, phospholipid, nucleic acid and phosphoprotein fraction was investigated.

Materials and Methods

The strains used were cultured in 250-ml. Erlenmeyer flasks containing peptone-tryptone medium (Slater, 1952). The initial pH was set at 7.6 and the length of incubation at 27° C. ±1° was 48 hours for log phase animals or 96 hours for those in the stationary phase. Each flask was initially inoculated with 10,000–20,000 cells.

Since conjugation in *Tetrahymena* can be induced by starvation in distilled water, the following procedure was used to produce masses of conjugating pairs. They were removed from the peptone medium by gentle centrifugation, washed twice with glass-distilled water and placed in glass-distilled water for 12 to 16 hours. They were then washed once again to free them from excretory products and energy sources, and reconcentrated. This was the preliminary procedure followed in all of the experiments.

The isotope used was P^{32} obtained as H_3PO_4 in weak HCl from the Oak Ridge National Laboratory. Specific activities of stock solutions averaged 60,000 mc./gm. The final concentration was 0.2 μc./ml. except where otherwise indicated. Radioactive organisms in the aliquots used for counting purposes were washed by centrifugation in construction chamber centrifuge tubes (Slater, 1957), the supernatant solutions being removed with micropipettes. Samples were then quantitatively transferred to 2.0-ml. volumetric test tubes and final volumes were adjusted to 2.0 ml. with distilled water. This volume was used to maintain constant geometry for counting. Finally, the samples were quantitatively transferred to 10-ml. plastic tubes.

Radiation counts were made with a deep-well scintillation detector and a TMC scaler (Model SG-2A) for all of the experiments except those having to do with the influence of population density and the phosphate distribution studies. For these later studies a Nuclear Chicago Ultra-Scaler was used. In all cases, 10-minute counts were taken, corrected for background, and calculated on a count/minute/animal basis. A self absorption study showed that corrections for this factor could be ignored.

For the conjugation studies, the two mating types after starvation were brought to equal optical densities by means of a Lumetron (Model 400) colorimeter at a wave-length of 650 mμ (Red). This made it possible to control the number of organisms used (Elliott, 1949). Equal concentrations of these solutions were then mixed, usually to a final volume of 100 ml. The P^{32} was then added, and this mixture was then distributed to 125-ml. Erlenmeyer flasks in 10-ml. aliquots. Samples were taken at this point for direct cell counts.

To test for P^{32} leakage, animals mixed for the induction of conjugation were allowed to take up the isotope for 9½ hours and then washed. Aliquots of the exposed animals in isotope-free distilled water were then counted for radioactivity at two-hour intervals.

With the individual mating types, the procedures were essentially the same as those outlined above for studying uptake during conjugation. Here, of course,

the mating types were not mixed, and direct counts of animals were made of each of the types after first adjusting them to approximately the same population density.

Since population density has been shown to influence ion uptake in experiments with Co^{60} (Slater, 1957), the influence of concentration of organisms on P^{32} uptake in each mating type was investigated. For this study, cultures of each mating type were grown for two days in 250-ml. Erlenmeyer flasks containing 25 ml. of stock peptone medium. The cells were then harvested by mild centrifugation ($100 \times g$ for 45 seconds) and after washing twice with glass-distilled water were suspended in 50 ml. of water. This constituted the highest population density. Dilutions from this population at approximately 3.5×10^6 cells were then prepared. Radioactive phosphorus was introduced to the extent of 0.10 μc./ml. After nine hours, the cells were washed free of external radioactivity and by successive centrifugation were concentrated to 2.0-ml. quantities for counting in the scintillation detector.

To determine the intracellular distribution of phosphorus in each mating type, cultures were grown for 48 hours in 250-ml. Erlenmeyer flasks, each containing 100 ml. of stock peptone medium. The protozoans were then removed and washed by means of further centrifugation with glass-distilled water. The suspensions were pooled and then diluted to 10 ml. with distilled water. Aliquots were removed for counts of radioactivity. Samples were also removed for cell counts.

The procedure used for the isolation of the general classes of phosphorus compounds was modified from Moraczewski and Kelsey (1948), and approximately 2×10^6 animals were used for each determination. Cell suspensions were initially placed in 15-ml. conical centrifuge tubes and 10 ml. of 15% trichloroacetic acid were added per 5 ml. of cell suspension. These were shaken for 15 minutes and then centrifuged at $150 \times g$ for 5 minutes. This thoroughly packed the sediment and resulted in the production of a clear supernatant liquid containing the acid-soluble fraction. The volume of liquid was recorded and 2.0 ml. were removed for radioactivity counts. The remaining liquid was then removed. One-half ml. of distilled water was added to the residue and after shaking for 15 minutes, 10 ml. of 3:1 alcohol-ether were added. This was then allowed to stand overnight, shaken 15 minutes and centrifuged 5 minutes. The soluble phospholipids present in the clear supernatant were then removed by pipette, the total liquid volume recorded, and 2.0-ml. aliquots removed for determinations of radioactivity.

Nucleic acid phosphorus was determined by adding 3 ml. of distilled water, shaking 15 minutes, boiling two minutes, and then adding 10 ml. of 10% trichloroacetic acid, followed by boiling again for 10 minutes. After cooling, the suspension was centrifuged 5 minutes. The dissolved nucleic acids were then removed quantitatively and 2.0-ml. aliquots taken for counts.

The determination of phosphoprotein phosphorus was made by adding 7 ml. of 0.2 N NaOH to the residue remaining after removal of the dissolved nucleic acids. This was then centrifuged, the total volume recorded, and aliquots were taken for radioactivity referable to phosphoprotein phosphorus in the solution.

Results

P^{32} uptake during conjugation. At relatively low population densities (about 11,000 animals/ml.) there was a significantly increased uptake of P^{32} six hours

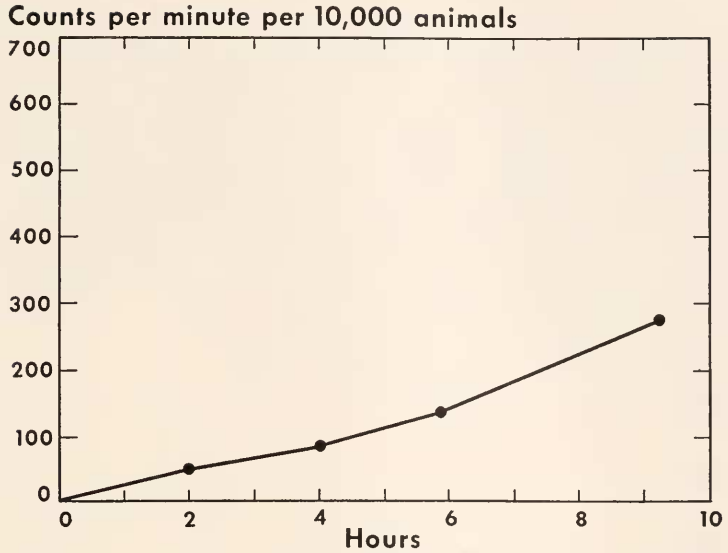

FIGURE 1. Uptake of radioactive phosphorus in *Tetrahymena* at low population densities. Maximum conjugation 30%. Average concentration of animals 11,000/ml.

after mixing the mating types for conjugation (Fig. 1). Feulgen staining of cultures after six hours indicated that the crescent stage of micronuclear division was prominent in all of the conjugating pairs. Prezygotic, meiotic divisions occurred between six and nine hours. At this relatively low population density, however,

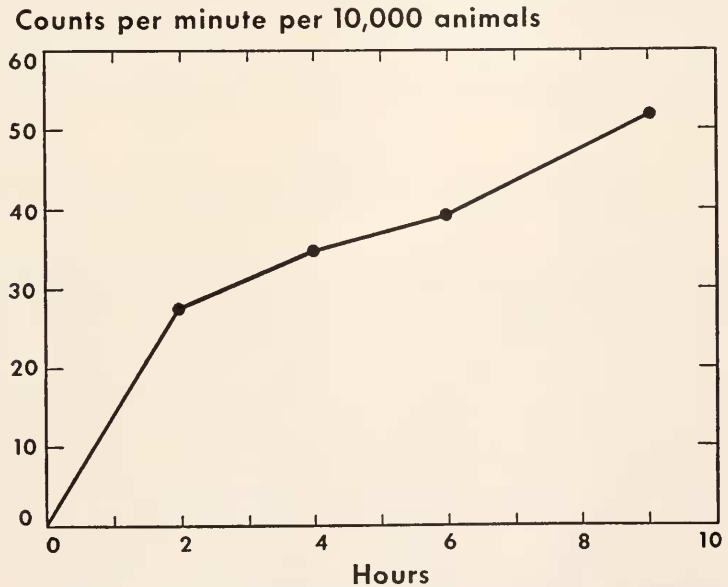

FIGURE 2. Uptake of radioactive phosphorus in *Tetrahymena* at high population densities. Maximum conjugation 60%. Average concentration of animals 236,000/ml.

conjugation at nine hours was less than 30% and synchrony of division was also low.

At higher population densities (Fig. 2) (236,000 animals/ml.) the rate of uptake of P^{32} remained constant at 3×10^{-4} cpm/animal/hr. after two hours. This was less than one-seventh that found when lower population densities were used. Up to 60% conjugation occurred when population densities were greater than 200,000 cells/ml. Further studies have also shown that population densities higher than about 260,000 cells/ml. result in reduced conjugation. A differential rate of uptake as seen with lower populations may have been masked by a superimposed population density effect, however, since it was not possible to correlate any uptake rate difference with a particular nuclear stage.

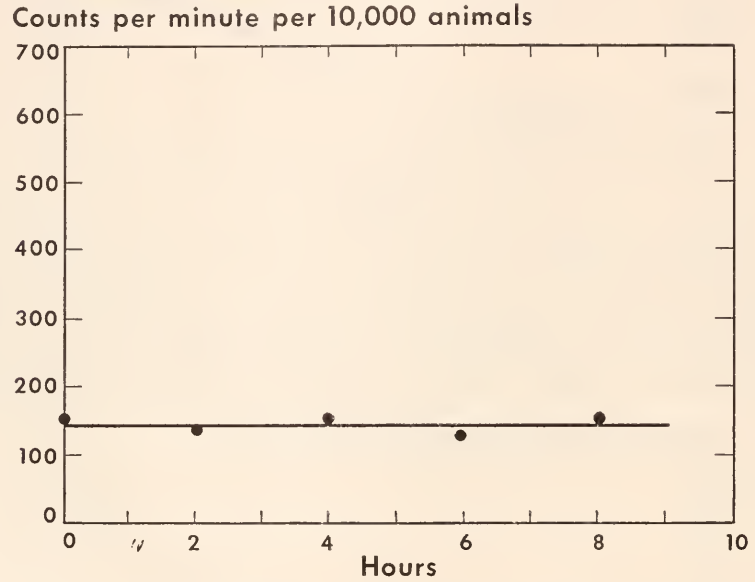

FIGURE 3. P^{32} retention in *Tetrahymena* when placed in distilled water after incubation with radioactive phosphorus. Concentration of animals 54,000/ml.

Leakage of P^{32} from conjugating Tetrahymena. The results of experiments in which all supernatant P^{32} was washed from conjugating animals, where radioactivity was measured at intervals, showed that leakage of phosphate from the ciliates could be eliminated as a possible source of error in the conjugation experiments. As Figure 3 shows, there was no loss of labeled phosphate from animals exposed to P^{32} when they were later placed in distilled water for periods up to eight hours.

P^{32} uptake in the individual mating types. When the individual mating types, after a starvation period, were tested for the uptake of P^{32} in distilled water, it was found that there was a significant difference between the two. The population densities for mating type I varied in the different experiments from 44,000 animals/ml., while those for mating type II varied from 34,000 animals/ml. to 244,000 animals/ml. Errors in these counts amounted to less than 5%.

It is evident that at these population densities mating type II takes up con-

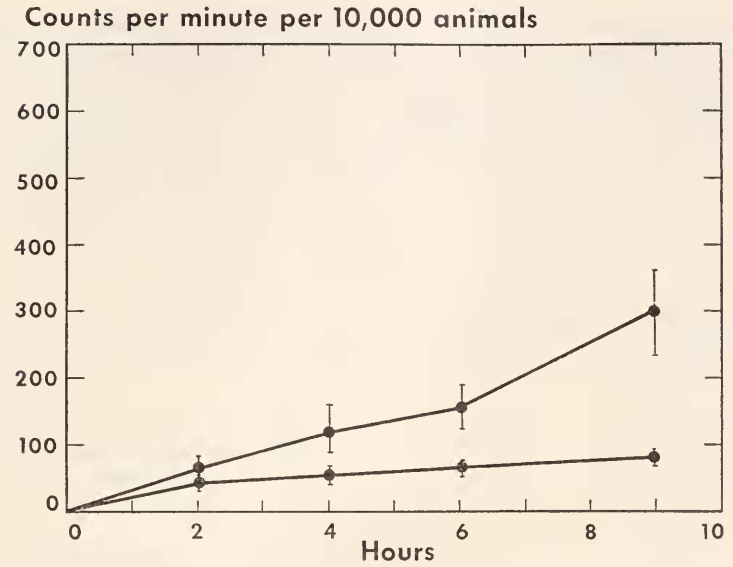

FIGURE 4. P^{32} uptake in the individual mating types. Mating type I. Lower curve. Average concentration: 100,000 animals/ml. Mating type II. Upper curve. Average concentration: 106,000 animals/ml. Standard deviation indicated.

siderably more P^{32} than mating type I and shows also an increased rate of uptake at six hours (Fig. 4). Mating type I, on the other hand, typically showed a slow, constant rate of uptake. It can readily be seen that, by taking the average of these two curves, there would still be an increased rate of uptake at six hours.

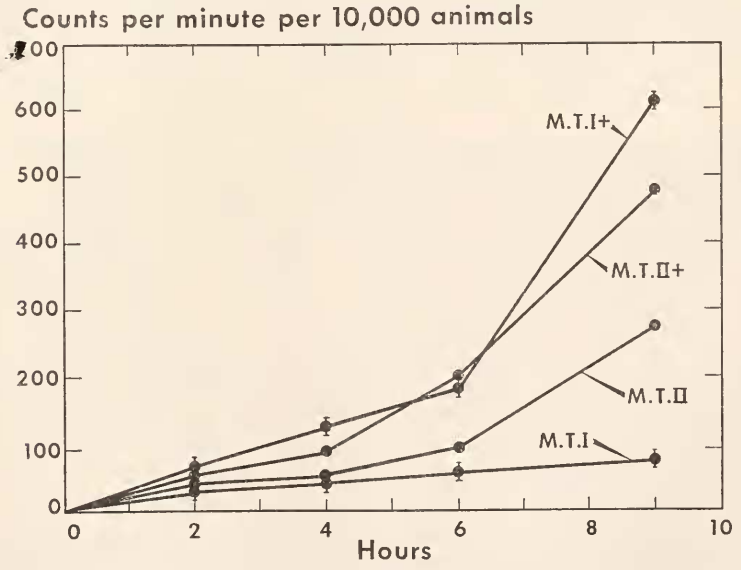

FIGURE 5. Influence of glucose on P^{32} uptake in individual. Population density, mating type I: 61,000/ml.; mating type II: 42,000/ml. Plus signs indicate cultures containing glucose.

Effect of glucose on P^{32} uptake in the individual mating types. After the usual starvation and washing procedure, glucose was added simultaneously with labeled phosphate to the distilled water into which the individual mating types were placed. This was done to determine the effect on P^{32} uptake of an added energy source as compared with the uptake resulting from the endogenous metabolism of the washed animals. The curves in Figure 5 again show the six-hour lag phase. In this instance, however, the presence of glucose was found to result in an initially greater rate of phosphate uptake, after which both mating types exhibited a sharply accentuated increased accumulation.

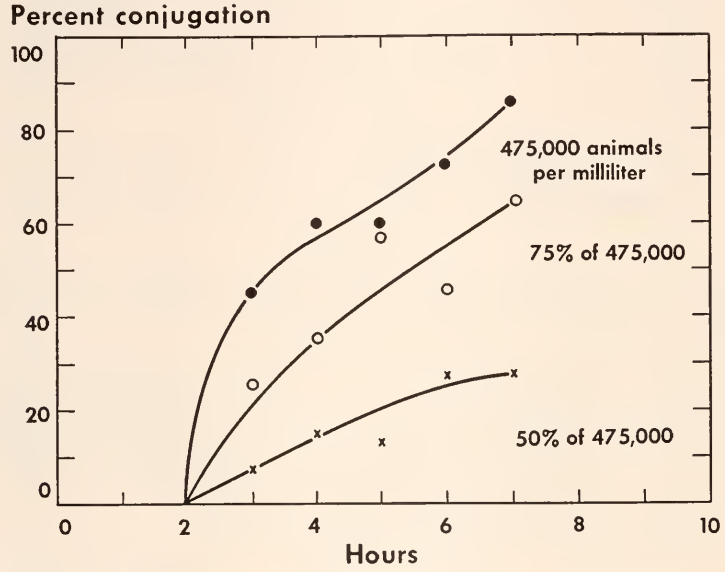

FIGURE 6. Effect of population density on rate of induction of conjugation.

Influence of population density on conjugation. It was suspected early in this investigation that the population densities used influenced the degree to which conjugation occurred. This was found to be the case, but other factors were involved, however. Cultures were found to be more fragile as they became older and since 96-hour cultures were used here in order to obtain large numbers of animals, fragmentation during centrifugation became a problem. Gentle centrifugation, just sufficient to concentrate the organisms, resulted in no fragmentation and under these conditions the subsequent percentages of conjugation were the greatest. Thus, after several hours, as much as 84% of the population were conjugating when population densities of 475,000 cells/ml. were used. Both maximal amounts of conjugation and conjugation rates within the population were found to be dependent upon the cellular densities (Fig. 6). It was found that conjugation rates were again strongly influenced by population densities and that densities higher than 260,000 cells/ml. produced considerable inhibition of conjugation.

Effect of population density on phosphate accumulation in the individual mating types. Population density (Table I) was discovered to influence the extent to

which P^{32} was accumulated by either mating type. With either type, large population densities strongly inhibited ion accumulation by the individual cells. At the lower population density levels, however, mating type II accumulated more than ten times as much phosphate than did mating type I.

Accumulation of P^{32}. For comparative purposes, the number of atoms of P^{32} taken up by each mating type was calculated.

TABLE I

Influence of population density on P^{32} uptake in Tetrahymena

Population	Total CPM	Uptake $\times 10^{-4}$/cell in 9 hrs.	Uptake $\times 10^{-7}$/ cell/min. CPM
3,585,000	3,788	10.6	29
1,792,500	2,002	11.2	21
896,250	1,397	15.6	29
448,125	2,572	57.4	106
224,063	11,319	505.1	935
Mating Type #2			
3,275,000	4,752	14.5	26
1,637,500	3,299	20.2	37
818,750	16,196	197.8	366
409.375	30,145	736.4	1,364

In medium containing 0.0374×10^7 atoms/volume equivalent to the average volume of one organism, mating type I was found to accumulate 7.2×10^7 atoms/organism, while mating type II accumulated 27.6×10^7 atoms/animal. When glucose was added to the medium, however, mating type II accumulated 44.1×10^7 atoms/animal while mating type I accumulated 56.0×10^7 atoms/animal.

TABLE II

Distribution of radioactive phosphorus in Tetrahymena Variety IV

Fraction	Mating Type #1 %	Mating Type #2 %
Acid-Soluble	7.6	19.5
Phospholipid	1.9	10.8
Nucleic Acid	11.7	21.6
Phosphoprotein	51.7	53.1

Intracellular distribution of phosphorus. Slightly more than one-half of the phosphorus in each mating type (Table II) was found to be localized in the phosphoprotein fraction. The nucleic acid fractions accounted for 11.7% of the phosphorus in mating type I and 21.6% in type II. Differences in the percentage of phospholipid phosphorus and acid-soluble phosphorus were the most apparent, however. Nearly five times as much phospholipid phosphorus occurred in type II as compared to that found in type I while about twice as much acid-soluble phosphorus was present in type II in comparison with that found in type I.

Discussion

In their study of radiophosphate uptake in *Paramecium multimicronucleatum,* Evans and Pendleton (1952) found that feeding paramecia accumulate roughly 20 times more activity than the surrounding medium. Unfed paramecia accumulated less than one-half the radioactivity. This is in contrast to our results which show that starving *Tetrahymena* concentrates P^{32} to an extent of 450 times that present in the medium. Among protozoans, unfed amoebae have been found to concentrate more than 50 times as much P^{32} than that contained in the surrounding substrate (Mazia and Hirshfield, 1950). Evans and Pendleton (1952) found also that older paramecia cultures took up P^{32} at a more rapid rate than young cultures. This was not evident in the case of *Tetrahymena* where 48-hour cultures and 96-hour cultures appear to take up the isotope at about the same rate.

Mazia and Hirshfield (1950) found in their experiments on nucleate and enucleate cells that enucleate fragments took up considerably less P^{32} than nucleated cells. Whether the nucleus influenced phosphorus metabolism in the cytoplasm directly without handling the phosphorus itself or whether this was an indirect, long-term nuclear function is not known. In any case, we might expect a very definite nuclear effect on cytoplasmic P^{32} uptake in a process such as conjugation.

The control of conjugation is difficult. Experimental conditions must be optimum for maximum conjugation and little is known about this optimum. It is known that the mating types must first be washed free of nutrients in order to induce conjugation and that a starvation period appears to be necessary (Elliott and Hayes, 1953). Population density, however, is undoubtedly important in inducing mating in this animal.

When low concentrations of animals were mixed for the induction of conjugation, the rate of P^{32} accumulation after six hours increased 122% over the preceding phase. Nuclear stains at this time revealed the presence of the crescent stage of division in conjugating pairs. This observation at first led to the conclusion that chromosomal reduplication resulting from possible preceding DNA synthesis (Swift, 1950) was probably mainly responsible for the increased ion uptake. However, the low percentage of conjugation (maximum 30%) and probably low degree of synchrony make it unlikely that conjugation *per se* was solely responsible for the differential ion uptake. Further ion accumulation studies with separated individual mating types showed that biphasic accumulation occurred.

It is of interest to note that McDonald (1958) has demonstrated a doubling of DNA in the macronuclei of *Tetrahymena pyriformis* H. after this strain was placed in an environment which does not support growth.

When large populations (over 200,000/ml.) were mixed for mating, inducing a high percentage of conjugation with a correspondingly higher degree of synchrony, the differential uptake was not observed. The effect of numbers of organisms on ion accumulation as observed in the case of non-conjugating animals (Slater, 1957) may have complicated and obscured any biphasic accumulation here. Thus, we were faced with the problem of obtaining a high percentage of fairly synchronous conjugation wherein large populations were needed with measurable P^{32} uptake.

Since only mating type II showed the biphasic uptake of radiocative phosphorus, while type I was typified by a slow constant rate of uptake, it would seem that, in the conjugation experiments with low population densities and a correspondingly

low percentage of conjugation, mating type II is probably responsible for the biphasic curve.

Of many possible explanations, the six-hour lag phase, recurring in many experiments, may represent a time of synthesis for an enzyme system to incorporate the P^{32}, or a time in which more acceptor sites for the element are created. The slow, constant uptake in mating type I might lead to the conclusion that this organism is more inactive metabolically. Under identical culture conditions, though, mating type I grows faster than type II, as indicated by colorimetric measurements (Elliott, 1949).

Significantly higher rates of P^{32} uptake were obtained in the presence of sugar. Sacks (1948) proposed that the cell membrane actively participates in phosphate transfer by the action of surface enzymes on the formation of hexose monophosphates, which by their spatial relationship to the cell surface may more easily enter. Once within, intracellular phosphatases split off the phosphorus. In this connection, Elliott and Hunter (1951) have demonstrated the presence of phosphatases in *Tetrahymena,* proposing their position to be either intracellular or at the membrane surface. Fennell and Degenhardt (1957) have located such phosphatases intracellularly in the same organism.

Notable, also, is the accentuated increase (about 215% over Phase I) in the rate of uptake of P^{32} at six hours in the types exposed to glucose. Possibly, this carbohydrate, in complementing the nutrient-free distilled water, may lead to the formation of more acceptor molecules for P^{32} than could be formed by the endogenous metabolism of the organism without the benefit of glucose. This might account for the very sharp increase of P^{32} uptake after the lag phase of synthesis.

Little difference was noted in regard to the localization of radioactive phosphorus in the phosphoprotein fractions in either mating type, but differences in the quantities of phospholipid phosphorus and acid-soluble phosphorus were quite apparent. The five-fold occurrence of phospholipid phosphorus in mating type II is probably correlated with a greater membrane permeability to this ion. The greater amount of acid-soluble phosphorus in this mating type is also probably concerned with glycolytic forces important in this process.

To evaluate the results of these experiments, various aspects of the isotope technique should be taken into consideration (Comar, 1955). In particular, possible physiological effects from the isotope-based radiation should be taken into account.

Not only should the amount of external radiation be considered, but also the degree of isotope accumulation or incorporation, which may have a greater effectiveness than otherwise anticipated. This accumulation of radioactivity may be important as a radiation hazard in *Tetrahymena* since in some of these experiments the organism accumulated large amounts of the isotope. Radiation effects were probably minimal, though, because of the short time intervals employed. Also, at the isotope concentrations used (0.1 μc.–0.2 μc.) no overt effects were noticed when compared to the controls. Since *Tetrahymena* does not appear to be noticeably influenced by radiation dosages less than about 5×10^5 roentgens (Elliott and Slater, 1951), it is unlikely that the isotope levels used were significant. Imperceptible nuclear or membrane changes may, of course, have occurred, though.

The isotope effect resulting in a preferential rate of incorporation of isotopes

differing in mass and mobility into biological systems may also be considered here.

These effects may be of significance only in experiments involving diffusion-like processes based on organic reactions, but even then the magnitude of the effect is usually less than 5% and may pass undetected in most biological experiments. Since P^{32} and P^{31} are essentially alike in their biological reactions the isotope effect can probably be neglected here.

The type of radiation of an isotope is important especially in regard to the counting instrumentation used. The scintillation detector used, though more adapted for gamma counting than for beta rays, was sensitive enough to register the hard beta rays in a reasonably efficient manner. Concentrated suspensions of *Tetrahymena* plus P^{32} yielded counts essentially the same as similar volumes of water plus the isotope.

As commented on before, care was taken to keep the geometry of the samples to be counted as constant as possible. This was especially necessary because of the extreme sensitivity of the scintillation detector to low-energy, scattered radiation.

In order to demonstrate a significant relationship between P^{32} uptake and chromosomal reduplication by the method we have chosen, as was an original intent of this study, many animals would have to be brought to nuclear stages as synchronously as possible.

Although almost 100% conjugation with good synchrony can sometimes be obtained in depression slides (Elliott and Hayes, 1953) the volumes used are too small for our methods. Zeuthen's (1954) methods for the induction of synchronous mitosis in *Tetrahymena* by means of alternate thermal shocks might prove useful for producing near-identical nuclear conditions in the production of synchronous conjugation, but thus far this synchronous mitosis has been induced only in amicronucleate strains (Zeuthen, 1956, private communication). It also seems apparent that the individual strains behave quite differently when treated in this manner, with a lesser degree of synchrony obtainable with strain E., for instance.

Constant conditions of centrifugation were employed to minimize cell breakage or stress during harvesting and washing, since Evans and Pendleton (1952) have demonstrated a leakage of P^{32} from paramecia washed by centrifugation. This loss did not seem to occur with *Tetrahymena*. Experiments designed to discover such leakage showed that P^{32} was completely retained over an eight-hour period.

Summary

1. *Tetrahymena* in distilled water containing P^{32} concentrated the isotope to an extent of 450 times that contained in comparable volumes of water after nine hours.

2. The accumulation of P^{32} by individual *Tetrahymena* varied with population density. With large populations, the amount of phosphate accumulated per cell was sharply reduced, whereas with low population densities larger quantities were accumulated. More than ten times as much P^{32} was found in mating type II than in mating type I.

3. The induction of conjugation was found to be strongly influenced by the amount of cellular debris present. The greatest amount of conjugation was obtained by the use of large population densities with gentle centrifugation. It was suspected also that age as influencing cell fragility was important here.

4. Mating type II showed a significantly increased rate of P^{32} accumulation six hours after addition of this isotope in distilled water, while mating type I was found to concentrate P^{32} at a slow constant rate.

5. Three times as much phosphate was accumulated during nine hours in the presence of glucose as when in distilled water alone with either mating type.

6. Although a biphasic accumulation was noted with low population densities prepared for the induction of conjugation, this was probably primarily because of an increased uptake by mating type II. Similar information was not obtained with large population densities, possibly because of the numbers effect.

7. Nearly five times as much phospholipid phosphorus was found in mating type II as compared to mating type I. This appears to be correlated with the greater ion accumulation ability found in type II. Only about twice as much acid-soluble phosphorus was found in type II compared with type I, however.

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